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TOPICAL REVIEW

Mitochondrial dynamics, mitophagy and cardiovascular disease

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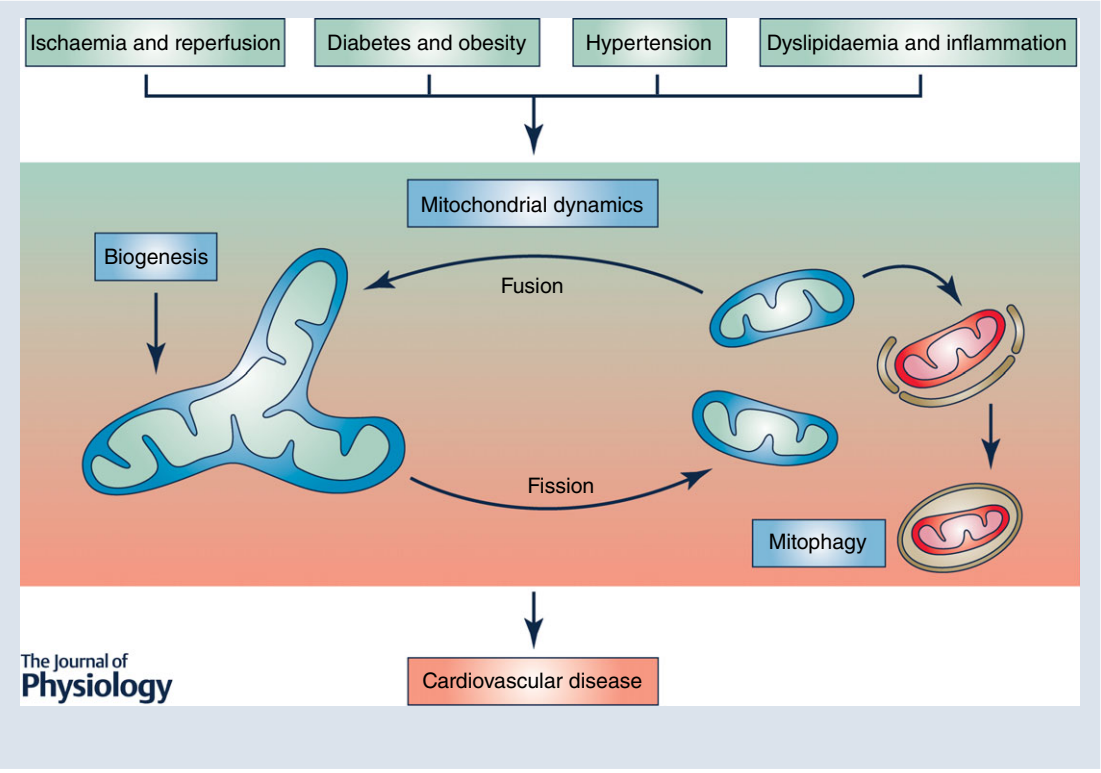

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Abstract Cardiac hypertrophy is often initiated as an adaptive response to haemodynamic stress or myocardial injury, and allows the heart to meet an increased demand for oxygen. Although initially beneficial, hypertrophy can ultimately contribute to the progression of cardiac disease, leading to an increase in interstitial fibrosis and a decrease in ventricular function. Metabolic changes have emerged as key mechanisms involved in the development and progression of pathological remodelling. As the myocardium is a highly oxidative tissue, mitochondria play a central role in maintaining optimal performance of the heart. 'Mitochondrial dynamics', the processes of mitochondrial fusion, fission, biogenesis and mitophagy that determine mitochondrial morphology, quality and abundance have recently been implicated in cardiovascular disease. Studies link mitochondrial dynamics to the balance between energy demand and nutrient supply, suggesting that changes in mitochondrial morphology may act as a mechanism for bioenergetic adaptation during cardiac pathological remodelling. Another critical function of mitochondrial dynamics is the removal of damaged and dysfunctional mitochondria through mitophagy, which is dependent on the fission/fusion cycle. In this article, we discuss the latest findings regarding the impact of mitochondrial dynamics and mitophagy on the development and progression of cardiovascular pathologies, including diabetic cardiomyopathy, atherosclerosis, damage from ischaemia–reperfusion, cardiac hypertrophy and decompensated heart failure. We will address the ability of mitochondrial fusion and fission to impact all cell types within the myocardium, including cardiac myocytes, cardiac fibroblasts and vascular smooth muscle cells. Finally, we will discuss how these findings can be applied to improve the treatment and prevention of cardiovascular diseases.

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Abstract figure legend Mitochondria play an essential role in maintaining optimal performance of the heart. Mitochondrial dynamics (processes of fusion and fission, mitochondrial biogenesis and mitophagy) determine mitochondrial morphology, quality and abundance. All these processes participate in the development and progression of cardiovascular pathologies, including diabetic cardiomyopathy, atherosclerosis, damage from ischaemia-reperfusion, cardiac hypertrophy and decompensated heart failure.

Abbreviations $\Delta\Psi_m$, mitochondrial membrane potential; DRP1, dynamin-related protein 1; FIS1, mitochondrial fission 1 protein; I/R, ischaemia/reperfusion; KO, knockout; MFF, mitochondrial fission factor; MFN, mitofusin; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OPA1, optic atrophy protein 1; PDGF, platelet-derived growth factor; PINK1, PTEN-induced putative kinase 1; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; VSMCs, vascular smooth muscle cells.

Mitochondrial dynamics as a therapeutic target in cardiovascular disease

Maintenance of mitochondrial function and integrity is crucial for normal cell physiology, particularly in cells with high energy demands. This is particularly certain in the heart, where mitochondria occupy approximately 30% of the total cell volume – and produce an astounding 6 kg of ATP per day through oxidative phosphorylation – in order to sustain cardiac mechanical function (Hall *et al.* 2014). In addition to ATP production, mitochondria regulate cell death and survival by integrating a range of cellular signals; they are also the primary source of reactive oxygen species (ROS), which can trigger oxidative stress, thereby affecting cell survival and death (Pellegrini & Scorrano, 2007).

Although mitochondria are often depicted as isolated organelles, they actually form highly dynamic networks whose structure and distribution have a potential effect on metabolic function. The nature of these intricate networks depends on the balance between the opposing processes of mitochondrial fission and fusion (Westermann, 2002; Parra *et al.* 2011). In mammalian cells, the primary regulators of mitochondrial fusion are dynamin-related GTPases termed mitofusins (MFN1 and MFN2) and optic atrophy protein 1 (OPA1). Conversely, mitochondrial fission 1 protein (FIS1) and the dynamin-related protein 1 (DRP1) are involved in mitochondrial fission (Ong & Hausenloy, 2010; Parra *et al.* 2011).

Given the unique and highly dynamic configuration of mitochondria in the adult heart, the effects of changes

in mitochondrial morphology in this organ are complex. Indeed, emerging data suggest that the dynamics of mitochondria are relevant to various aspects of cardiovascular biology: these include cardiac development, responses to ischaemia/reperfusion (I/R) injury, heart failure, type 2 diabetes mellitus (T2DM) and apoptosis. In this article, we review how changes in mitochondrial morphology, and mitochondrial fission and fusion proteins, impact heart function.

Mitochondrial fusion and fission in cardiovascular cells

Fusion and fission machinery. The molecular machinery that controls the processes of mitochondrial fusion and fission is highly regulated. As noted above, mitochondrial fusion in mammals is controlled by MFN1 and MFN2, proteins situated in the outer mitochondrial membrane, and OPA1, which is located in the inner mitochondrial membrane (Cipolat *et al.* 2004) (Fig. 1). MFN1 and MFN2 are necessary for mitochondrial fusion, and cells lacking either of these GTPases display a significant decrease in the fusion process. Also, the proteins are partially redundant in their function; thus when MFN1 expression is decreased, mitochondrial fusion can be rescued by MFN2 overexpression and vice versa (Chen & Chan, 2005; Chan, 2006). Additionally, MFN2 has been implicated in several other physiological functions, such as modulation of energetic processes, endoplasmic reticulum–mitochondria coupling, and regulation of mitophagy, a process through which mitochondria are engulfed by autophagosomes and delivered to lysosomes for degradation (Pich *et al.* 2005; Zorzano *et al.* 2009; Chen & Dorn, 2013).

The second component regulating mitochondrial fusion is OPA1, a transmembrane protein tightly associated with the mitochondrial inner membrane. The transcript coding for OPA1 undergoes alternative splicing, giving rise to eight variants that are expressed in a variety of patterns across different tissues (Delettre *et al.* 2001). Moreover, OPA1 also undergoes proteolytic processing, resulting in short (s) and long (l) isoforms, both of which are necessary for mitochondrial fusion (Song *et al.* 2007). Also, uncoupling of mitochondrial membrane potential ($\Delta\Psi_m$) leads to OPA1 processing (Griparic *et al.* 2007) through the action of the OMA1 metalloprotease (Head *et al.* 2009). Additional studies have implicated other proteases in the processing of OPA1, including presenilin-associated rhomboid-like (PARL) protein, paraplegin and the mAAA protease complex ATPase family gene-3, yeast-like-1 (AFG3L1) (McBride & Soubannier, 2010).

Mitochondrial fission in mammalian cells is controlled by the GTPases DRP1, FIS1, mitochondrial fission factor (MFF), and mitochondrial dynamic proteins of 49 and 51 kDa (MiD49/51) (Hall & Hausenloy, 2012) (Fig. 1). DRP1, like dynamins, functions as a mechanoenzyme, serving to constrict the mitochondrion physically, an early step in fission. Noteworthy, DRP1 lacks a mitochondrial destination sequence; as a result, it requires that FIS1 be located in the mitochondrial outer membrane to form the fission complex (Bossy-Wetzel *et al.* 2003). However, silencing FIS1 in mammalian cells has little effect on DRP1 translocation to mitochondria (Lee *et al.* 2004). In that context, MFF appears to be the protein acting as the DRP1 mitochondrial receptor (Gandre-Babbe & van der Bliek, 2008). Reduction of MFF levels induces mitochondrial elongation and decreases DRP1 translocation to mitochondria (Gandre-Babbe & van der Bliek, 2008). Likewise, MiD49 and MiD51 are involved in the mammalian fission machinery (Otera *et al.* 2010). Currently, it is widely accepted that multiple receptors

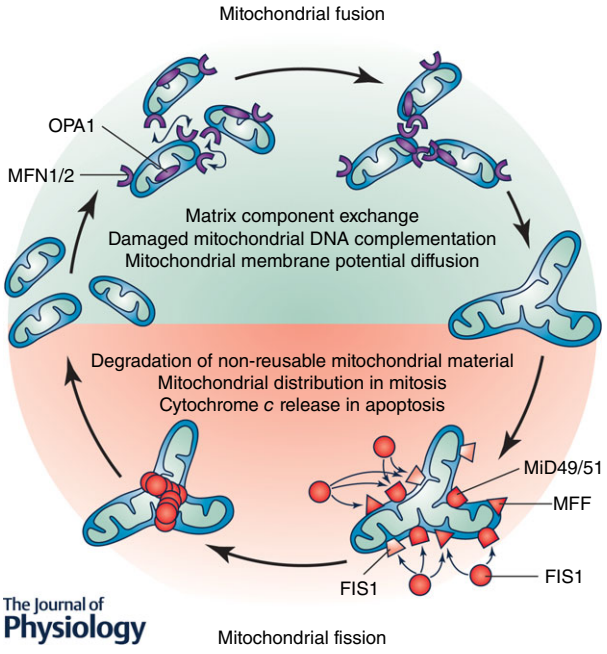

Figure 1. Complex interplay between mitochondrial fusion and fission supports cell homeostasis
Mitochondrial dynamics sustains a balance between the processes of mitochondrial fusion and fission to maintain the proper function of this complex organelle. Mitochondrial fusion is regulated by mitofusin (MFN) 1 and 2 and optical atrophy protein 1 (OPA1). This allows for exchange of material (matrix components, damaged mitochondrial DNA), as well as promoting a balance in bioenergetic properties (e.g. mitochondrial membrane potential). On the other hand, DRP1 and its adapter proteins FIS1, MFF and MiD49/51 control mitochondrial fission. This process is necessary for various processes throughout the life of the cell, such as redistribution of mitochondria in mitosis, as well as release of cytochrome c during cell death by apoptosis. Moreover, mitochondrial fission is required for selective mitochondrial degradation (mitophagy).

are capable of recruiting DRP1 to mitochondria to induce fission.

DRP1 activity is also modified by post-translational modifications. The kinases Cdk1/cyclin B (Taguchi *et al.* 2007) and CaMKIα (Han *et al.* 2008) increase DRP1 fission activity; conversely, protein kinase A phosphorylation decreases DRP1 function (Chang & Blackstone, 2007). Specifically, this phosphate residue can be removed by the Ca^{2+}–calmodulin-dependent phosphatase calcineurin, promoting mitochondrial fission (Cereghetti *et al.* 2008).

Mitochondrial fusion, fission and mitophagy work together to maintain mitochondrial and cellular homeostasis. Perturbations in mitochondrial dynamics that alter the normal balance between fission and fusion in either direction can lead to the accumulation of damaged and inefficient organelles. Mitochondrial fission is essential for maintenance and repair as it facilitates the removal of damaged components by partitioning them to a daughter that can then be targeted for removal and degradation by mitophagy. However, excessive mitochondrial fission and mitophagy can compromise the metabolic capacity of a cell (Twig & Shirihai, 2011). Thus, maintaining an appropriate balance in the fission/fusion cycle is essential.

As will be discussed in detail later, emerging key players in the regulation of mammalian mitophagy are the serine/threonine kinase PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin, which selectively promote the degradation of impaired mitochondria (Narendra *et al.* 2008). In healthy mitochondria, PINK1 is preferentially imported to the mitochondrial internal membrane, where it is cleaved by the protease PARL. Although Parkin-induced mitophagy has been shown to be dependent on the activity of PINK1, this process depends on DRP1-mediated mitochondrial fission (Tanaka *et al.* 2010; Fischer *et al.* 2012). A full understanding of the regulatory mechanisms involved in these processes has yet to be developed, as well as their connection with the onset of cardiovascular diseases.

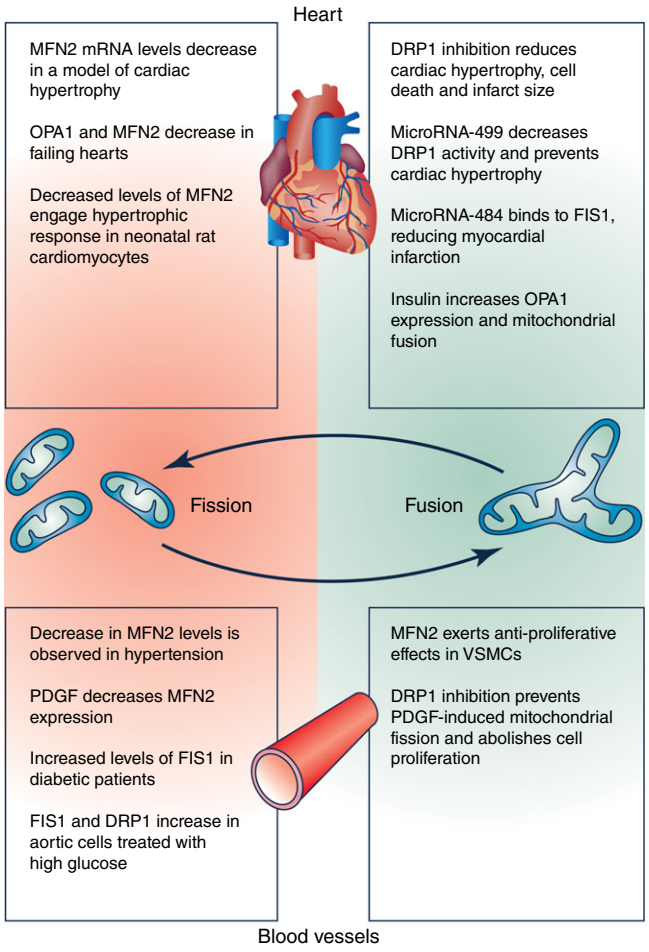

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Figure 2. Mitochondrial dynamics and cardiovascular diseases
Summary of important molecular events in cardiovascular diseases associated with mitochondrial fission (red zone) and therapeutic interventions or molecular processes related to mitochondrial fusion and linked with an improved cardiovascular function (green zone).

Cardiac myocytes. In cardiac myocytes, unremitting mitochondrial function is essential, owing to the high energy demands of this ever-pumping muscle. Interestingly, the distribution and metabolic function of these organelles varies depending on the developmental stage of the myocardium (Lopaschuk & Jaswal, 2010). In neonatal cardiac myocytes, the heart preferentially obtains its energy from glycolysis and glucose oxidation (Lopaschuk *et al.* 1991). During this period, mitochondria manifest a characteristic reticular distribution in the cytosol that allows them to move freely. However, in adult cardiac myocytes, energy derives mainly from the oxidation of fatty acids (Stanley *et al.* 2005), and the motility of mitochondria is restricted.

Mitochondria in adult cardiac myocytes localize within three subcellular distributions: interfibrillar, subsarcolemmal and perinuclear (Makinde *et al.* 1998; Piquereau *et al.* 2012; Ong *et al.* 2013). Interfibrillar mitochondria are relatively uniform in size and shape. They align longitudinally between the myofibrils and are situated at places where Ca^{2+} release occurs. Their main function is to provide ATP for muscle contraction. Subsarcolemmal and perinuclear mitochondria are less well organized and have greater variation in their shape and size (Iglewski *et al.* 2010). These mitochondria are involved in the transport of metabolites and electrolytes through the sarcolemma, and perinuclear mitochondria probably have a role in nuclear transcription (Shimada *et al.* 1984; Ong & Hausenloy, 2010).

Although mitochondrial fusion and fission are most evident in neonatal cardiac myocytes due to the features

described above (Chen *et al.* 2011), there is strong evidence that these processes are important in the adult cardiac tissue (Coleman *et al.* 1987; Delettre *et al.* 2001; Santel *et al.* 2003). For example, the proteins involved in fusion and fission are highly expressed in the adult heart (Delettre *et al.* 2001; Santel *et al.* 2003). Ultrastructural analysis of murine left ventricle subjected to physical training reveals the presence of giant mitochondria, thought to be the result of multiple fusion events (Coleman *et al.* 1987).

More evidence for the importance of mitochondrial dynamics in the heart comes from loss-of-function studies of proteins involved in the process. Papanicolaou *et al.* demonstrated that *Mfn1* knockout (KO) mice harbour small and spherical mitochondria within cardiac myocytes, although cardiac function remained normal (Papanicolaou *et al.* 2011*b*). In contrast to MFN1 deletion, *Mfn2* KO mice harbour enlarged mitochondria, which protects cardiac myocytes from proapoptotic stimuli (Papanicolaou *et al.* 2011*a*). Cardiac myocytes isolated from murine models of an inducible ablation of *Mfn-1/2* harbour fragmented mitochondria with abnormal cristae (Papanicolaou *et al.* 2011*a*). These morphological changes were accompanied by alterations in mitochondrial respiration, mitophagy and mitochondrial biogenesis which ultimately culminated in cardiomyopathy (Chen *et al.* 2011; Papanicolaou *et al.* 2012).

Abnormalities in mitochondrial morphology were also observed in mice heterozygous for *Opa1*. In this model, large mitochondria with abnormal cristae were found. However, decreased expression of OPA1 did not alter mitochondrial respiration (Piquereau *et al.* 2012). Mitochondria within these cardiac myocytes also manifest increased ability to accumulate Ca^{2+} and are marked by a delayed opening of the mitochondrial permeability transition pore (mPTP), coupled with increased sensitivity to prolonged mechanical stress (Piquereau *et al.* 2012). Chen *et al.* also analysed cardiac myocytes from *Opa1*$^{+/-}$ mice and reported that mitochondria showed a pattern of abnormal cristae and disruption in mitochondrial organization (Chen *et al.* 2012). Alterations in functional parameters included increased production of ROS and a decrease in mitochondrial DNA (mtDNA) content, indicating that mitochondrial function was impaired.

Cardiac fibroblasts. Cardiac fibroblasts are the most abundant cell in the myocardium, accounting for 60–70% of the total population of cells. They are essential for the maintenance of the extracellular matrix (ECM) and hence, structural and mechanical properties of the heart (Porter & Turner, 2009). Cardiac fibroblast function is mediated by the differentiation of cardiac fibroblasts into myofibroblasts, cells with enhanced capacity for proliferation, migration and secretion (Porter & Turner, 2009). In addition, cardiac myofibroblasts are primarily reactive to mechanical stress, hormones, proinflammatory cytokines and vasoactive peptides (angiotensin II). In response, these cells synthesize and secrete ECM proteins, along with a variety of signalling molecules.

Despite all these relevant functions in the heart, only a single study has emerged exploring mitochondrial dynamics in cardiac fibroblasts, Samant *et al.* showed that SIRT3 binds directly to OPA1 and activates it by deacetylation (Samant *et al.* 2014). Adult *Sirt3* KO cardiac fibroblasts manifest reduced mitochondrial fusion and loss of $\Delta\Psi_m$ (Samant *et al.* 2014). Given the importance of mitochondrial dynamics in cardiac function and metabolism, additional research is warranted, focusing on establishing links between mitochondrial dynamics and the function of cardiac fibroblasts and myofibroblasts.

Vascular smooth muscle cells. The principal function of vascular smooth muscle cells (VSMCs) is the regulation of vascular tone and consequently blood pressure and blood flow. Unlike cardiac myocytes, these cells are highly plastic and undergo reversible changes in phenotype in response to environmental stimuli (Jay *et al.* 2008; Martin-Garrido *et al.* 2013). Differentiated VSMCs have a contractile phenotype, which is characterized by little proliferation, minimal secretion of extracellular matrix, and expression of specific contractile proteins, such as smooth muscle myosin heavy chain, smooth muscle α-actin and calponin (Owens, 1995). However, when a vessel is damaged, these cells transform into a synthetic phenotype in which the cells proliferate and migrate towards the injury site (Johnson *et al.* 2001). These events are accompanied by increased secretion of ECM components and expression of other proteins, such as vimentin and tropomyosin-4 (Owens, 1995; Abouhamed *et al.* 2003).

VSMC proliferation can be induced by several factors, including platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), angiotensin II and endothelin-1 (Janakidevi *et al.* 1992; Pukac *et al.* 1998; Watanabe *et al.* 2014). Proliferation is inhibited by other molecules, such as transforming growth factor-β (TGF-β) and heparin (Björkerud, 1991; Lindner *et al.* 1992).

Recently, a relationship has been proposed between the VSMC proliferative phenotype and mitochondrial dynamics (Chalmers *et al.* 2012; Marsboom *et al.* 2012). For instance, pulmonary artery smooth muscle cells from hypertensive mice manifest hyperproliferation along with a fragmented mitochondrial phenotype (Marsboom *et al.* 2012). Additional studies show that PDGF promotes mitochondrial fragmentation and changes the metabolic profile of VSMCs, increasing fatty acid oxidation and decreasing glucose oxidation (Salabei & Hill, 2013). PDGF induces mitochondrial fragmentation reducing MFN2 levels (Salabei & Hill, 2013), without changing the levels of other proteins that regulate mitochondrial dynamics,

such as DRP1, FIS1 and OPA1. Using a pharmacological inhibitor of DRP1 (mdivi-1) to inhibit mitochondrial fission, PDGF stimulation of VSMC proliferation was prevented as well as changes in glucose and fatty acid oxidation, suggesting that mitochondrial fission is required for these processes (Chalmers *et al.* 2012; Salabei & Hill, 2013). Thus, mitochondrial fission is an important event in the development of the synthetic phenotype of VSMCs (Salabei & Hill, 2013).

Mitochondrial fission is also necessary for oxygen-dependent constriction of the ductus arteriosus. Prolonged exposure of ductus arteriosus smooth muscle cells (DASMCs) to oxygen, significantly increased the levels of DRP1 and triggered mitochondrial fragmentation, thereby increasing oxygen consumption and oxidative metabolism (Hong *et al.* 2013). When DRP1 was inhibited, proliferation of DASMCs and closure of the ductus arteriosus were each prevented (Hong *et al.* 2013).

In summary, although research in this area is limited, there is some background information regarding the relationship between VSMC phenotypes and mitochondrial dynamics. This relationship is probably significant, given the established role of the VSMC synthetic phenotype in the development of diseases such as pulmonary hypertension and atherosclerosis (Lacolley *et al.* 2012; Marsboom *et al.* 2012).

Mitochondrial dynamics and cardiovascular disease

Cardiac hypertrophy and failure. Cardiac hypertrophy is a response triggered by a wide variety of stimuli, including haemodynamic overload, neurohormonal activation and ischaemia. Thought to be initially adaptive, this response involves structural, morphological and functional changes in cardiac myocytes, including increases in cell size, thereby provoking an overall growth in heart mass (Frey *et al.* 2004; Barry *et al.* 2008; Hill & Olson, 2008).

Hypertrophic growth begins as a compensatory process that normalizes wall stress and oxygen demand. However, prolonged exposure to disease-related stimuli leads to pathological cardiac myocyte growth, cell death and heart failure (Burchfield *et al.* 2013). Throughout, mitochondrial metabolism remains essential for adequate myocardial pump function, because cardiac myocytes require vast amounts of energy in this state in order to maintain contractile performance, Ca^{2+} homeostasis and ion transport (Huss & Kelly, 2005).

In adult cardiac myocytes, mitochondria are tightly packed between myofibrils (Vendelin *et al.* 2005) and it has been reported that this subpopulation of mitochondria do not undergo mitochondrial dynamics events (Beraud *et al.* 2009). However, there is evidence from models of both cardiac hypertrophy (Fang *et al.* 2007) and heart failure (Chen *et al.* 2009) for robust mitochondrial dynamics-related changes. Chen *et al.* described small and fragmented mitochondria in both human and rat models of heart failure, which were associated with decreased OPA1 levels (Chen *et al.* 2009). Other studies in neonatal rat cardiac myocytes exposed to phenylephrine to induce hypertrophy (Fang *et al.* 2007), as well as *in vivo* models of cardiac hypertrophy, also described decreases in *Mfn2* mRNA levels (Fang *et al.* 2007).

Calcineurin is an essential regulator of cardiac hypertrophy and heart failure (Heineke & Molkentin, 2006) and participates in the regulation of mitochondrial fission by DRP1 dephosphorylation (Cereghetti *et al.* 2008). Wang *et al.* showed that both the A and B isoforms of the calcineurin catalytic subunit are direct targets of microRNA (miR)-499, increasing DRP1 phosphorylation at residue Ser^{656}, thereby reducing mitochondrial fission (Wang *et al.* 2011). Interestingly, miR-499 transgenic mice manifested a decline in hypertrophic parameters assessed by heart/body weight ratio, cardiac myocyte cross-sectional area, collagen content, heart chamber dimensions and cardiac function after ischaemia–reperfusion (I/R). Conversely, knockdown of endogenous miR-499 aggravated maladaptive cardiac remodelling (Wang *et al.* 2011).

We recently reported that noradrenaline (norepinephrine) triggers mitochondrial fission in a calcineurin- and DRP1-dependent manner (Pennanen *et al.* 2014). Adenovirus-mediated expression of dominant-negative DRP1 prevented mitochondrial fission and hypertrophic cardiac myocyte growth in response to noradrenaline. Furthermore, an adenovirus-expressed antisense sequence to MFN2 was sufficient to increase mitochondrial fission and elicit a hypertrophic response in cultured cardiac myocytes (Pennanen *et al.* 2014) (Fig. 2). Consistent with these results, Papanicolaou *et al.* reported that *Mfn2*-deficient mice display modest cardiac hypertrophy accompanied by a slight functional deterioration (Papanicolaou *et al.* 2011*a*). In a more recent work, wild-type mice were subjected to ascending aortic banding to generate heart failure and then treated with the DRP1 inhibitor mdivi-1. Left ventricular dysfunction was ameliorated by treatment with mdivi-1, and these effects were associated with decreased expression of the autophagy markers LC3 and p62 (Givvimani *et al.* 2012).

The findings described in this review suggest a predominant role for mitochondrial fission in pathological cardiac remodelling. However, in a DRP1-mutant mouse model that alters protein interactions and prevents mitochondrial fission events, elongated mitochondrial networks associated with reduced levels of mitochondrial enzyme complexes, cardiac ATP depletion and heart failure were observed (Ashrafian *et al.* 2010). One possible explanation is that disruption of mitochondrial fission prevents appropriate mitochondrial quality control,

leading to accumulation of damaged and dysfunctional mitochondria. This idea, on which we elaborate later, is consistent with observations in a *Parkin* knockout *Drosophila* model, in which disruption of mitophagy triggered accumulation of enlarged mitochondria in heart tubes and dilated cardiomyopathy (Bhandari *et al.* 2014).

Diabetic cardiomyopathy. Cardiovascular complications are the primary cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM) and mitochondrial dysfunction has been implicated in the pathogenesis and almost all the complications observed in T2DM (Bugger & Abel, 2014). An important contributory factor linked to the increased risk of cardiovascular disease is hyperglycaemia-induced mitochondrial oxidative stress which can cause cellular injury and dysfunction (Green *et al.* 2004). Consistently, *in vitro* data from H9c2 cells suggest that hyperglycaemia induces mitochondrial fragmentation (Yu *et al.* 2006). In this report, H9c2 cells (a myoblast cell line, derived from embryonic rat heart) exposed to sustained hyperglycaemia manifested mitochondrial fragmentation and mitochondrial ROS accumulation resulting in cell death by apoptosis (Yu *et al.* 2006). Importantly, these events were prevented by over-expression of DRP1K38A, a fission-inactive mutant of DRP1, suggesting that this process was DRP1 dependent (Yu *et al.* 2006).

Moreover, in a recent article, Watanabe *et al.* presented evidence from H9c2 cells for a functional interaction between DRP1 and ROS promoting mitochondrial dysfunction and inhibition of insulin signalling. This effect was partially overcome when cells were treated with the superoxide dismutase (SOD) mimetic TMPyP (Watanabe *et al.* 2014). In a different model, Makino *et al.* reported that coronary endothelial cells from murine diabetic hearts displayed mitochondrial fragmentation associated with reduced OPA1 levels and increased levels of DRP1. In this work, although mitochondrial fragmentation was abolished by 4 weeks of pre-treatment with antioxidants, protein levels of OPA1 and DRP1 were not restored (Makino *et al.* 2010). In aggregate, these data suggest a role for oxidative stress as a mediator of mitochondrial fragmentation, a notion that has been confirmed by the fact that oxidative stress induces additional mitochondrial fragmentation in coronary endothelial cells (Makino *et al.* 2010).

Following this same line of investigation, exposure of neonatal cardiac myocytes to high levels of glucose (35 mM) increased mitochondrial fragmentation and reduced mitochondrial membrane potential ($\Delta\Psi_m$) and electron transport chain activity. High glucose treatment decreased mitochondrial OPA1 and MFN1 protein levels whereas FIS1 levels were significantly increased. Overexpression of OPA1 was able to reverse some of the negative effects of high glucose on cardiac myocytes (Makino *et al.* 2011). Atrial tissue sections from T2DM patients show an increase in mitochondrial fragmentation and a reduction in MFN1 protein levels (Montaigne *et al.* 2014).

Recently, our group demonstrated a link between mitochondrial dynamics and insulin's impact on cardiac myocyte metabolism (Parra *et al.* 2014). Insulin treatment of either cardiac myocytes or skeletal muscle cells increased OPA1 levels and triggered mitochondrial fusion, enhancing ATP production and the rate of oxygen consumption (Parra *et al.* 2014) (Fig. 2). These findings are consistent with those reported by Quirós *et al.* (2012) who showed that alterations in the proteolytic processing of OPA1 in *Oma1* knockout mice leads to insulin resistance, impaired glucose homeostasis, and altered thermogenesis. *Oma1*$^{-/-}$ mice develop metabolic defects similar to those seen with high-fat feeding, highlighting the importance of OPA1 and the relative stoichiometry of its l and s isoforms for maintaining mitochondrial function (Griparic *et al.* 2007; McBride & Soubannier, 2010). In our experimental model, the effect of insulin on mitochondrial metabolism was impaired in cells deficient for OPA1 and MFN2 (Parra *et al.* 2014). These findings are also consistent with the clinical observation of impaired mitochondrial function and diminished levels of OPA1 and MFN2 in individuals with diabetes (Kelley *et al.* 2002; Lowell & Shulman, 2005; Zorzano *et al.* 2009).

Mitochondrial dynamics directly influence pancreatic function as well. In *ob/ob* mice, OPA1 levels decrease in pancreatic islet cells before the onset of diabetes (Keller *et al.* 2008). Silencing *Opa1* in pancreatic β cells using a Cre-loxP system yields similar results (Zhang *et al.* 2011). β cells lacking OPA1 maintained normal mtDNA copy number, but electron transport chain complex IV levels and activity were significantly reduced, leading to impaired glucose stimulation of ATP production and insulin secretion.

Whether mitochondrial fission contributes to the cardiac dysfunction associated with diabetic cardiomyopathy is unknown. Similarly, it is not known whether mitochondrial fusion acts as a direct mediator of mitochondrial metabolism and cardiac function. However, recent evidence suggests that mitochondrial fragmentation is a 'starting point' of numerous events participating in cardiac metabolic disease (Montaigne *et al.* 2014). Contractile dysfunction is observed in T2DM patients but not in 'metabolically healthy' obese patients at early stages of the insulin-resistant state (Montaigne *et al.* 2014). Based on this, we speculate that declines in ventricular performance occurring in the transition from obesity to diabetes mellitus are caused, at least in part, by deterioration of cardiac myocyte mitochondrial function. Further studies are required to test whether modulation

of mitochondrial dynamics could emerge as a means to enhance mitochondrial function and ultimately cardiac performance.

Myocardial infarction and ischaemia/reperfusion. Ischaemic heart disease is the leading cause of death and disability worldwide (Writing Group Members *et al.* 2006). Its clinical manifestations derive from the detrimental effects of acute I/R on the myocardium. During myocardial infarction, cardiac myocyte metabolism is severely perturbed owing to deprivation of oxygen and nutrients coupled with intracellular acidification (Thygesen *et al.* 2008). In this context, susceptibility to, and recovery from, I/R injury are critically dependent on mitochondrial function. Mitochondrial dysfunction in response to acute I/R and the subsequent opening of the mPTP during reperfusion are critical determinants of cell death (Davidson *et al.* 2014). Therefore, preservation of mitochondrial health and the prevention of mPTP opening during acute I/R are two important therapeutic targets for cardioprotection.

Whether changes in mitochondrial dynamics occur in the heart in response to I/R was first addressed by Brady *et al.* who described extensive mitochondrial fission in HL-1 cells (a murine atrial myocyte-derived cell line) in response to sustained ischaemia, changes which persisted during reperfusion and were inhibited by the p38MAPK inhibitor SB203580 (Brady *et al.* 2006). Furthermore, Ong *et al.* reported that inhibition of mitochondrial fission by mdivi-1 protects cardiac myocytes from I/R injury in both *in vitro* and *in vivo* models (Ong *et al.* 2010).

Inhibition of DRP1 with mdivi-1 increased the proportion of adult cardiac myocytes with elongated mitochondria and protected them against simulated I/R by inhibiting mPTP opening and resulted in decreased infarct size (Ong *et al.* 2010). In this same context, we recently reported that DRP1 inhibition reduces I/R injury through declines in cardiomyocyte oxygen dependence while increasing proton leak-associated oxygen consumption (Zepeda *et al.* 2014). Moreover, Sharp *et al.* reported that DRP1 inhibition has therapeutic benefits even when administered after ischaemia (Sharp *et al.* 2014). Inhibition of DRP1 dephosphorylation at Ser^{637} in a Langendorff model conferred cardioprotection by preserving mitochondrial morphology and cytosolic calcium levels, and diminishing cell death (Sharp *et al.* 2014). Using a different strategy, Din *et al.* suppressed DRP1 translocation to mitochondria (and thereby mitochondrial fission) in simulated I/R through the over-expression of Pim1, implicating this kinase as another potential mechanism of cardioprotection (Din *et al.* 2013). Finally, in a more recent study, a particular peptide inhibitor of DRP1, P110, was employed, revealing that inhibition of mitochondrial fission at reperfusion reduces myocardial infarct size and prevents adverse left ventricular remodelling post-myocardial infarction in the adult rat heart (Disatnik *et al.* 2013).

Mechanisms described to this point suggest that cytosolic Ca^{2+} overload and ROS may be contributory factors to mitochondrial fragmentation observed during I/R. In this way, mitochondrial fragmentation interferes with cellular respiration and can result in ROS overproduction. Consistently, Plotnikov *et al.* (2008) showed that pre-treatment with an antioxidant could prevent ischaemia-induced mitochondrial fission (Plotnikov *et al.* 2008). Interestingly, in this study, insulin was also reported to prevent mitochondrial fragmentation, although the mechanism was not investigated. These findings agree with our recent report describing cardiac myocyte mitochondrial fusion triggered by insulin (Parra *et al.* 2014).

Other works have uncovered beneficial effects of inhibiting mitochondrial fission using cardiac microRNAs (Wang *et al.* 2011, 2012). In the first study, Wang *et al.* reported that modulation of miR-499 levels affects apoptosis and the extent of myocardial infarction and cardiac dysfunction induced by I/R by targeting calcineurin-mediated DRP1 activation (Wang *et al.* 2011). However, two other groups have expressed miR-499 in mouse hearts and found that, rather than being protective, the intervention predisposed to heart failure (Shieh *et al.* 2011; Dorn *et al.* 2012). Also, calcineurin A has not yet been fully confirmed as a valid *in vitro* target of miR-499 in the myocardium (Dorn *et al.* 2012). Thus, the conclusion about miR-499 and its potentially cardioprotective properties remains unclear.

More recently, a second paper also associated a miR with mitochondrial dynamics and heart protection during I/R. In this report, Wang *et al.* showed that miR-484, a microRNA which binds to the amino acid coding region of FIS1 transcripts can repress FIS1 expression and thereby inhibit mitochondrial fission (Wang *et al.* 2012). These investigators also reported that FoxO3a transactivates miR-484 expression. FoxO3a transgenic or knockout mice exhibit, respectively, a high or low level of miR-484 and a reduced or enhanced mitochondrial fission, apoptosis and myocardial infarction (Wang *et al.* 2012).

Reduced levels of OPA1 have been reported in samples from human hearts with ischaemic cardiomyopathy (Chen *et al.* 2009). However, the role of the mitochondrial fusion proteins (MFN1, MFN2 and OPA1) as targets of cardioprotection remains in flux, as unexpected and contradictory findings have emerged. In HL-1 cells, Ong *et al.* reported that over-expression of MFN1 or MFN2 prevented the opening of the mPTP and reduced cell death after I/R (Ong *et al.* 2010). However, Papanicolaou *et al.* in a parallel study showed that small interfering RNA (siRNA) knockdown of MFN2 delayed mPTP opening, thus rendering cardiac myocytes more susceptible to ROS

(Papanicolaou *et al.* 2011*a*). Similarly, partial genetic ablation of *Opa1* has been shown to delay mPTP opening, although the effect on acute I/R has not been studied (Piquereau *et al.* 2012). In summary, the interplay between mitochondrial fusion proteins and I/R is complex and requires additional in-depth analysis.

Atherosclerosis. Atherosclerosis is a chronic inflamma tory disease that predisposes to I/R injury to the heart, brain and other tissues and is a significant risk factor for premature death (Libby, 2002). In this complex disorder, arterial endothelium expresses elevated levels of adhesion molecules, which promote monocyte infiltration, differentiation and transformation into highly active lipid-loaded foam cells, accompanied by migration of VSMCs (that also have the potential to become foam cells) toward the intima (Libby, 2002). Mitochondrial generation of ROS is the most thoroughly documented relationship between mitochondria and vascular disease (Cho *et al.* 2013; Wang & Tabas, 2014). Conversely, ROS molecules also target this organelle, where mitochondrial DNA is probably the most sensitive cellular target (Anan *et al.* 1995; Wallace, 1999). Emerging evidence indicates that mitochondrial DNA damage can directly promote atherosclerosis. Ballinger *et al.*, working with human aortic specimens and a murine model of early atherogenesis (apolipoprotein E null mice), found that mitochondrial DNA damage correlated with the extent of atherosclerosis in both models (Ballinger *et al.* 2002). In the case of apolipoprotein E null mice, it was associated with deficiency of manganese SOD, a mitochondrial antioxidant enzyme, with early increases in mitochondrial DNA damage, and a phenotype of accelerated atherogenesis (Ballinger *et al.* 2002).

Increased mitochondrial ROS production also causes endothelial dysfunction, along with proliferation and apoptosis of VSMCs and macrophages, with ensuing atherosclerosis lesion progression and possible plaque rupture (Madamanchi & Runge, 2007). Directly related to this, Shenouda *et al.* reported mitochondrial fragmentation and increased levels of FIS1 protein in venous endothelial cells from patients with T2DM, as well as increased abundance of FIS1 and DRP1 proteins in cultured human aortic endothelial cells incubated with high glucose medium (Shenouda *et al.* 2011). Altered mitochondrial dynamics were associated with increased mitochondrial ROS production, and silencing FIS1 or DRP1 expression with siRNA prevented high glucose-induced alterations in mitochondrial networks and ROS production (Shenouda *et al.* 2011).

In atherosclerosis, VSMCs develop a highly proliferative and synthetic phenotype in a process triggered by PDGF (Yu *et al.* 2001; Perez *et al.* 2010). Exposure of VSMCs to PDGF resulted in mitochondrial fragmentation and decreases in MFN2 protein levels (Salabei & Hill, 2013). Treatment of VSMCs with mdivi-1 inhibited PDGF-induced mitochondrial fragmentation and abolished increases in cell proliferation (Salabei & Hill, 2013). Collectively, these data demonstrate the importance of mitochondrial fission machinery in the pathogenesis of vascular disease.

Mitophagy in cardiovascular cells and its role in cardiovascular diseases

In response to various environmental stresses, cellular ATP synthesis can be disrupted. Mitochondria then become major producers of ROS, which causes cellular damage and can initiate the apoptotic pathway (Kubli & Gustafsson, 2012). However, cells have developed a defence mechanism against ROS production by aberrant mitochondria (Levine & Kroemer, 2012). Fundamental among these is the selective sequestration and degradation of dysfunctional mitochondria before they cause damage or trigger cell death, through a process called mitochondrial autophagy or mitophagy (Kubli & Gustafsson, 2012).

Macroautophagy, hereafter referred to as autophagy, is a highly conserved mechanism for the degradation of cytoplasmic components, including damaged organelles, toxic protein aggregates and intracellular pathogens (Mizushima *et al.* 2008; Gatica *et al.* 2015). Basal autophagy plays a key role in eukaryotic cells, degrading long half-life macromolecules and large supramolecular structures, including organelles such as mitochondria, peroxisomes and endoplasmic reticulum (Levine & Kroemer, 2012). Autophagy initiates with formation of the autophagosome, a double-membrane intracellular structure of reticular origin that engulfs cytoplasmic contents and ultimately fuses with lysosomes for cargo degradation. Materials degraded within these newly formed autolysosomes are recruited to anabolic reactions to maintain energy levels and provide macromolecules for the synthesis of higher-order structures (nucleic acids, proteins or organelles), thereby sustaining cell metabolism, homeostasis, and survival (Mizushima, 2007; Gatica *et al.* 2015). Initial phagophore formation requires the assembly of a complex consisting of BECLIN1, vacuolar protein sorting (VPS) 34 and VPS15 (Kang *et al.* 2011). Next, the expansion of the membrane is mediated by two ubiquitin-like conjugation systems, microtubule-associated protein 1 light chain 3 (LC3) and autophagy protein (ATG) 12-ATG5, that promote assembly of the ATG16L complex and the conjugation of LC3 with phosphatidylethanolamine (Mizushima *et al.* 1999; Kabeya *et al.* 2004). The phagophore expands until its borders fuse around its target(s), forming a structure with double membrane, called the autophagosome. Finally, the autophagosome fuses with a

lysosome and the contents are degraded by lysosomal enzymes (Mizushima *et al.* 2008).

The same stimuli that trigger mitochondrial-dependent cell death can trigger mitophagy (Kubli & Gustafsson, 2012). The balance between these two highly regulated processes will then determine whether a cell lives or dies. Understanding the role of mitochondria and mitophagy in various cardiovascular pathologies is currently an area of great interest. Ensuring the proper removal of dysfunctional mitochondria is critical as mitochondrial damage has been linked to diverse cardiovascular pathologies (Lavandero *et al.* 2015).

PINK1/Parkin mitophagy pathway. Mitophagy in mammalian cells is mainly regulated by the cytosolic E3 ubiquitin ligase Parkin (Kitada *et al.* 1998) and the outer mitochondrial membrane kinase PINK1 (Valente *et al.* 2004), two genes altered in Parkinson's disease. Upon mitochondrial impairment or loss of mitochondrial potential (for example using a mitochondrial uncoupler, such as CCCP), Parkin translocates from the cytosol (Fig. 3) to damaged or uncoupled mitochondria, promoting ubiquitination of several mitochondrial outer membrane proteins, such as MFN1, MFN2, VDAC, MIRO1, etc. (Chan *et al.* 2011). Parkin recruitment to damaged mitochondria is selective and requires the participation of PINK1 (Kawajiri *et al.* 2010; Narendra *et al.* 2010; Vives-Bauza *et al.* 2010). In healthy mitochondria, PINK1 is rapidly degraded by proteolysis and maintained at low levels. In the setting of mitochondrial damage, however, PINK1 cleavage is inhibited, allowing PINK1 accumulation selectively within damaged organelles (Matsuda *et al.* 2010). Thus, Parkin recruitment is specific to mitochondria that have high levels of accumulated PINK1.

PINK1 phosphorylates MFN2 and promotes Parkin translocation to mitochondria (Chen & Dorn, 2013) (Fig. 3). In mouse cardiac myocytes, MFN2 removal prevented depolarization-induced recruitment of Parkin to damaged mitochondria, triggering progressive cardiomyopathy (Chen & Dorn, 2013). This phenomenon highlights the relevance of the PINK1/Parkin pathway in the quality control of cardiac mitochondria. Indeed, PINK1 is essential for healthy heart function. *Pink1*$^{-/-}$ mice develop early left ventricular dysfunction and pathological cardiac hypertrophy (Billia *et al.* 2011). This phenotype is accompanied by increased oxidative stress and mitochondrial impairment in cardiac myocytes. Supporting these data, PINK1 protein levels are also notably reduced in advanced human heart failure (Billia *et al.* 2011).

On the contrary, *Parkin*-deficient mice manifest normal myocardial function (Kubli *et al.* 2013). *Parkin*$^{-/-}$ hearts harbour altered mitochondrial networks with small mitochondria, although mitochondrial function is unaffected. Despite this, *Parkin*$^{-/-}$ mice are more

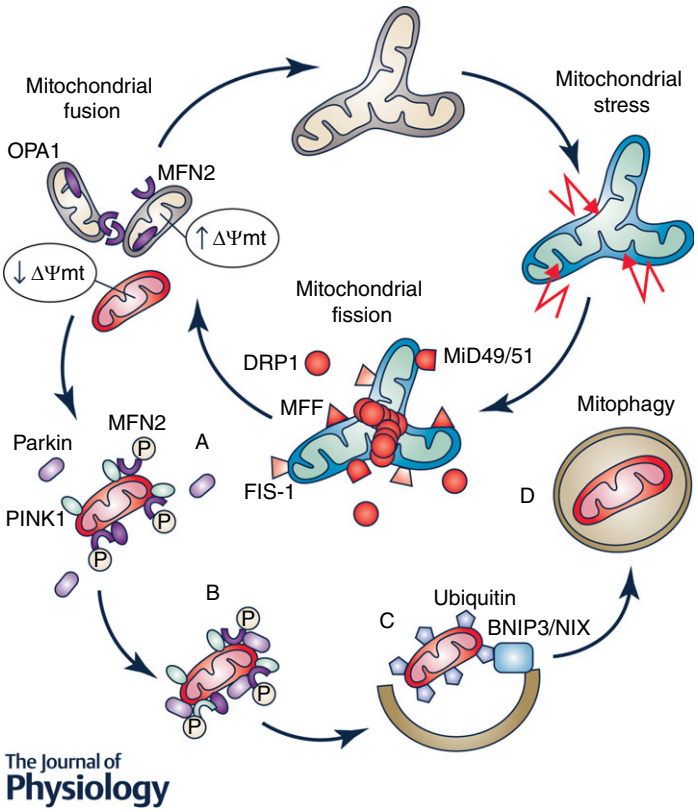

Figure 3. Mitophagy
The process of selective targeting of mitochondria by autophagy (mitophagy) is coordinated with mitochondrial fusion and fission to ensure proper mitochondrial quality control. Mitochondrial fission isolates damaged mitochondria (usually with low membrane potential) to initiate the process of mitophagy. Under conditions of low mitochondrial membrane potential, PINK1 kinase accumulates on a mitochondrion and phosphorylates MFN2 (A). Parkin E3 ligase binds phosphorylated MFN2 and localizes to the mitochondrion (B). To catalyse the ubiquitination of several proteins on the mitochondrial surface, various adapter proteins, such as BNIP3 and NIX, are associated with the autophagy machinery (C), ultimately facilitating elimination of mitochondrial content (D).

susceptible to myocardial infarction damage induced by coronary artery ligation (Kubli *et al.* 2013). Collectively, these data suggest a non-redundant function for PINK1 in mammalian cardiac tissue, in contrast to Parkin. This idea is supported by an interesting study from the Dorn group (Bhandari *et al.* 2014). In *Parkin* knockout mouse heart, there is a compensatory up-regulation of several Parkin-related E3 ubiquitin ligases of the RING families (Bhandari *et al.* 2014). Studying heart tubes of *Parkin*-KO *Drosophila* mutants (because, in contrast with mice, *Drosophila* lacks *Parkin*-redundant genes), Dorn *et al.* observed enlarged mitochondria accompanied by dilated cardiomyopathy. Parkin-deficient mitochondria are depolarized and generate enhanced ROS. This abnormal phenotype was rescued by suppressing mitochondrial fusion (by silencing MARF, the MFN2 *Drosophila* orthologue), normalizing mitochondrial morphology and function and preventing the cardiomyopathic phenotype (Bhandari *et al.* 2014). Additionally, a new study from the same group showed that Parkin mRNA and protein are present at very low levels in normal mouse hearts, but are upregulated after cardiac myocyte-specific deletion of the *Drp1* gene in adult mice). Thus, *Drp1* deficiency appears to trigger Parkin-dependent over-activation of mitophagy leading to a severe myopathic phenotype. The authors propose that DRP1 helps in maintaining mitochondrial quality control by promoting mitochondrial fission to segregate dysfunctional mitochondria that can then be targeted by mitophagy (Song *et al.* 2015). These data highlight the central role of mitochondrial dynamics in cardiac mitochondria quality control, ensuring proper elimination of damaged and dysfunctional organelles (Cao *et al.* 2015). In support of this notion, Sadoshima's group recently highlighted the participation of DRP1 in mitophagy. DRP1 down-regulation induces accumulation of damaged mitochondria due to suppressed mitochondrial autophagy (Ikeda *et al.* 2015).

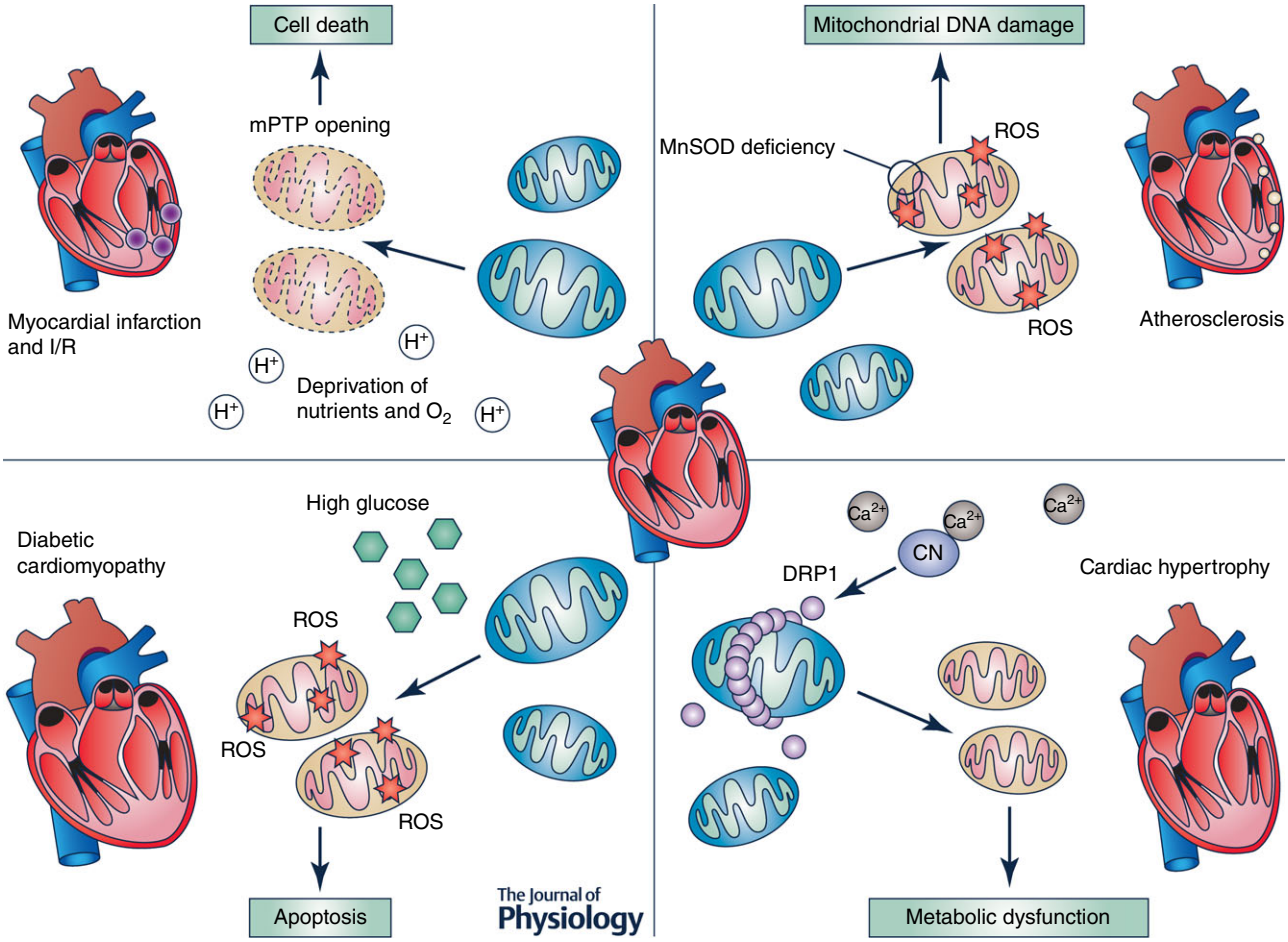

Figure 4. Mitochondrial dynamics and cardiovascular remodelling
Alterations in mitochondrial dynamics have a significant role in cardiovascular remodelling. Cardiac hypertrophy, diabetic cardiomyopathy, myocardial infarction and atherosclerosis share common pathological mechanisms and features related to fragmentation of the mitochondrial network. Mitochondrial morphological alterations finally lead to metabolic dysfunction, damage of mitochondrial components (mtDNA) and/or cell death.

Also, *Drp1*-CKO (cardiac myocyte-specific KO) mice develop mitochondrial and cardiac dysfunction and are prone to I/R injury (Ikeda *et al.* 2015; Song *et al.* 2015).

NIX and BNIP3 during cardiac mitochondrial clearance. In mammalian cells, NIX/BNIP3 and mitochondrial pro-apoptotic BH3-only domain protein (BNIP3) are involved in the selective mitochondrial clearance. The canonical function of BNIP3 and NIX in cardiac myocytes is to sense myocardial damage and initiate the apoptotic programme through activation of the pro-apoptotic proteins Bax and Bak (Youle & Strasser, 2008). Although BNIP3 and NIX perform similar functions regulating cardiac myocyte death, both are controlled by stimuli of a different nature. BNIP3 is up-regulated in myocardium during hypoxia (Regula, 2002), while NIX appears to be up-regulated in pathological cardiac hypertrophy (Gálvez *et al.* 2006).

Additionally, Dorn *et al.* (2010) showed that both BNIP3 and NIX are mitophagy regulators in adult heart. Mitochondrial distribution in *Bnip3/Nix* DKO hearts was markedly perturbed, and mitochondrial morphology manifested an atypical phenotype characterized by loss and dilatation of mitochondrial cristae. These results showed that BNIP3 and NIX play constitutive roles in the elimination of damaged cardiac mitochondria. However, under sustained stress conditions, such as hypoxia or pathological hypertrophy, BNIP3 and NIX trigger cardiac myocyte death.

Concluding remarks

Mitochondrial dynamics play a fundamental role in homeostasis of the cardiovascular system. This process is associated with important cellular functions such as metabolism and quality control. Specifically, the balance between mitochondrial fusion and fission is linked with mitochondria energy production. Given the never-ceasing mechanical demands on the cardiovascular system, provision of energy must be continuous, especially in the heart. Metabolic remodelling occurs in a diversity of cardiovascular diseases. Changes in mitochondrial dynamics can be directly linked to a decline in energy efficiency, as increased mitochondrial fission is associated with a decrease in ATP production. For example, mitochondrial dynamics are frequently altered in examples of either metabolic overload, such as in diabetes, or nutrient deprivation, such as in periods of prolonged ischaemia (Fig. 4). These changes in mitochondrial structure and function lead to an imbalance in energy production. It will be important for future research to fully define the mechanisms that trigger and carry out adaptive and pathological changes in mitochondrial dynamics (protein levels and function).

Of equal importance is the roles of mitochondrial dynamics and mitophagy in quality control and repair. It is of particular interest to investigate the participation of mitophagy in cardiovascular disease, as current evidence indicates that it can be both an essential protective mechanism when repairing damage and a pathological one when it progresses unrestrained. Thus, if mitophagy is to emerge as a useful therapeutic target, it will be critical to determine where and when manipulating mitophagy activity can prevent, counteract or reverse a specific cardiovascular disease.

In summary, we highlight here evidence for novel interventions related to mitochondrial dynamics with relevance to certain cardiovascular diseases. We speculate that further research into mitochondrial dynamics will uncover novel approaches to therapeutic manipulation of cardiac myocyte energetics, mitochondrial function and quality control.

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Additional information

Competing interests

None declared.

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